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# Downregulation of the Tem1 GTPase by Amn1 after cytokinesis involves both nuclear import and SCF-mediated degradation

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1 **Downregulation of the Tem1 GTPase by Amn1 after cytokinesis involves both**  
2 **nuclear import and SCF-mediated degradation**

3

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12 **ABSTRACT**

13 At mitotic exit the cell cycle engine is reset to allow crucial processes, such as cytokinesis  
14 and replication origin licensing, to take place before a new cell cycle begins. In budding  
15 yeast, the cell cycle clock is reset by a Hippo-like kinase cascade called Mitotic Exit  
16 Network (MEN), whose activation is triggered at spindle pole bodies (SPBs) by the Tem1  
17 GTPase. Yet, MEN activity must be extinguished once MEN-dependent processes have  
18 been accomplished. One factor contributing to switch off MEN is the Amn1 protein, which  
19 binds Tem1 and inhibits it through an unknown mechanism. Here, we show that Amn1  
20 downregulates Tem1 through a dual mode. On one side, it evicts Tem1 from SPBs and  
21 escorts it into the nucleus. On the other, it promotes Tem1 degradation as part of an SCF  
22 ubiquitin ligase. Tem1 inhibition by Amn1 takes place after cytokinesis in the bud-derived  
23 daughter cell, consistent with its asymmetric appearance in the daughter versus the  
24 mother cell. This dual mechanism of Tem1 inhibition by Amn1 **may** contribute to rapidly  
25 extinguish MEN activity once it has fulfilled its functions.

## 26 INTRODUCTION

27

28 The Mitotic Exit Network (MEN) is an essential kinase cascade that promotes mitotic exit  
29 and cytokinesis in budding yeast (Bardin and Amon, 2001; Simanis, 2003). MEN has a  
30 similar organisation as the Septation Initiation Network in fission yeast and the Hippo  
31 pathway in metazoans, in that it includes a Ste20-like protein kinase (Cdc15) that activates  
32 a LATS-NDR kinase (Mob1-Dbf20/Df20) (Hergovich and Hemmings, 2012). A major  
33 function of MEN is to promote the release from the nucleolus and activate the Cdc14  
34 phosphatase (Mohl et al., 2009; Shou et al., 1999; Visintin et al., 1999), i.e. the main CDK-  
35 counteracting phosphatase in budding yeast (Bouchoux and Uhlmann, 2011; Gray et al.,  
36 2003; Visintin et al., 1998).

37

38 MEN signalling is triggered by the Tem1 GTPase, which gets activated at microtubule-  
39 organizing centres or spindle pole bodies (SPBs) (reviewed in Scarfone and Piatti, 2015).  
40 During metaphase, Tem1 is present on both SPBs, albeit with a slight preference for the  
41 bud-proximal SPB. In anaphase it becomes markedly asymmetric, getting progressively  
42 concentrated on the bud-directed SPB (Bardin et al., 2000; Campbell et al., 2020; Caydasi  
43 and Pereira, 2009; Frascini et al., 2006; Molk et al., 2004; Monje-Casas and Amon, 2009;  
44 Pereira et al., 2000; Scarfone et al., 2015). In spite of this asymmetry, both SPB-bound  
45 pools of Tem1 seem to contribute to mitotic exit (Campbell et al., 2020).

46 Once active, Tem1 recruits to SPBs and activates the MEN kinase Cdc15, which in turn  
47 phosphorylates the MEN scaffold Nud1, thereby creating a phospho-docking motif for the  
48 Mob1-Dbf2 kinase. At SPBs, Cdc15 activates Mob1-Dbf2 that ultimately allows Cdc14  
49 nucleolar release and activation (Mah et al., 2001; Mohl et al., 2009; Rock and Amon,  
50 2011; Rock et al., 2013; Scarfone et al., 2015; Valerio-Santiago and Monje-Casas, 2011;  
51 Visintin and Amon, 2001).

52

53 How MEN signalling is extinguished after cytokinesis is an important, yet unanswered,  
54 question. Indeed, unscheduled Cdc14 phosphatase activity in G1 interferes with DNA  
55 replication (Bloom and Cross, 2007). Furthermore, Cdc14-independent MEN functions  
56 (Hotz et al., 2012; Meitinger et al., 2011; Meitinger et al., 2013; Oh et al., 2012) might also  
57 need to be turned off.

58 One mechanism that contributes to MEN downregulation after mitotic exit is the re-  
59 entrapment of Cdc14 in the nucleolus prompted by Cdc14 itself (Lu and Cross, 2010;  
60 Manzoni et al., 2010; Visintin et al., 2008).

61 Another actor that contributes to turn down MEN signalling after mitotic exit is the MEN  
62 antagonist Amn1 protein. Amn1 is expressed from mitotic exit to late G1 and accumulates  
63 specifically in the nucleus of the bud-derived daughter cell (Wang et al., 2003).

64 Asymmetric, cell cycle-dependent regulation of Amn1 expression requires the daughter-  
65 specific transcription factor Ace2 (Colman-Lerner et al., 2001) and Amn1 degradation in  
66 late G1 (Wang et al., 2003). In turn, Ace2 activation depends on MEN and Cdc14 (Brace  
67 et al., 2011; Sanchez-Diaz et al., 2012; Weiss et al., 2002). Thus, besides promoting its  
68 own re-entrapment, Cdc14 also triggers Amn1-dependent downregulation of MEN.

69 How exactly Amn1 antagonizes MEN signalling remains to be elucidated. Amn1 interacts  
70 physically with Tem1 and competes with Cdc15 for Tem1 binding (Wang et al., 2003).

71 However, higher levels of Tem1 are present in *amn1* $\Delta$  cells (Wang et al., 2003),  
72 suggesting that Amn1 could inhibit Tem1 function also by promoting its turnover.

73 Accordingly, in G1 Tem1 is present at low levels on SPBs (Campbell et al., 2020; Molk et  
74 al., 2004). Furthermore, Amn1 is an atypical F-box protein part of the SCF (Skp, Cullin, F-  
75 box-containing complex) ubiquitin ligase, driving proteolysis of Ace2 in most, but not all,  
76 laboratory strains (Fang et al., 2018).

77

78 Here, we show that Amn1 has a dual function in Tem1 downregulation after mitotic exit.  
79 On one side, it promotes Tem1 degradation as part of an SCF<sup>Amn1</sup> complex, while on the  
80 other it displaces Tem1 from SPBs and escorts it into the nucleus independently of SCF.  
81

## 82 RESULTS AND DISCUSSION

83

### 84 **Amn1 prompts asymmetric removal of Tem1 from the daughter cell SPB and** 85 **concomitant nuclear import after cytokinesis**

86 To study Tem1 localisation, we used a strain expressing the endogenous *TEM1* gene  
87 tagged at the C-terminus with yeast-enhanced GFP (yeGFP). This strain has a doubling  
88 time indistinguishable from that of the isogenic untagged strain, suggesting that the tag  
89 does not perturb Tem1 activity (Fig. S1A). By live cell imaging, we observed a transient  
90 and asymmetric relocalisation of Tem1-yeGFP into the nucleus of the bud compartment,  
91 which was visualised by the nuclear marker mCherry-Pus1 (98/98 cells, Fig. 1A). Tem1  
92 nuclear translocation occurred about 20 minutes after anaphase, and was accompanied  
93 by a decrease of Tem1 levels at the daughter-bound SPB. To better resolve the timing of  
94 Tem1 nuclear import, we filmed cells co-expressing Tem1-yeGFP and the mCherry-tagged  
95 septin Shs1. Tem1-yeGFP appeared in the nucleus of the bud 4 minutes (+/- 3 min; n=45)  
96 after septin ring disassembly, which marks the onset of cytokinesis (Lippincott et al., 2001;  
97 Tamborini et al., 2018), and persisted in the nucleus throughout the following G1 phase  
98 until appearance of a new septin ring (Fig. S1B). Thus, Tem1 undergoes an asymmetric  
99 accumulation in the nucleus of the daughter cell after cytokinesis until late G1.

100

101 Next we investigated the molecular bases of Tem1 nuclear import. We reasoned that  
102 Amn1 could be implicated in this process since it interacts with Tem1 and is concentrated  
103 in the daughter cell nucleus after mitotic exit (Wang et al., 2003). In agreement with our  
104 prediction, Tem1-yeGFP nuclear import was completely abolished in *amn1Δ* cells (93/93  
105 cells), and the protein persisted at both SPBs throughout the cell cycle (Fig. 1B).

106 Furthermore, *AMN1* deletion increased the levels of Tem1 at the daughter cell SPB from  
107 cytokinesis to the following G1 without affecting Tem1 levels at the mother cell SPB (Fig.  
108 1C).

109 Consistent with a role of Amn1 in downregulating Tem1 levels at SPBs specifically in G1,  
110 Tem1 levels were increased by more than twofold at SPBs of G1-arrested *amn1Δ* cells  
111 relative to their wild type counterparts (Fig. S1C).

112 Importantly, live cell imaging of cells co-expressing Amn1-sfGFP and Tem1-mScarlet-I  
113 showed that the two proteins concomitantly concentrate in the nucleus (104/109 cells, Fig.  
114 1D).

115 These data suggest that Amn1 could escort Tem1 into the nucleus of the daughter cell to  
116 reduce its active pool at the daughter-bound SPB after cytokinesis, i.e. once Tem1 has  
117 accomplished its essential functions.

118

### 119 **The SCF<sup>Amn1</sup> complex promotes Tem1 ubiquitination and degradation**

120 Amn1 is an atypical F-box protein that was recently shown to be part of an SCF (Skp,  
121 Cullin, F-box-containing) complex (Fang et al., 2018). In agreement with these data, we  
122 confirmed by co-immunoprecipitation experiments that Flag-tagged Amn1 (Amn1-3Flag)  
123 interacts with the HA-tagged cullin Cdc53 and Skp1 also in our strain background (W303,  
124 Fig. 2A-B, lane 3).

125 We then asked if Tem1 is associated with the SCF<sup>Amn1</sup> complex. First, we confirmed the  
126 association between Tem1-3HA and Amn1-3Flag by co-immunoprecipitations (Fig. 2C,  
127 lane 2). Second, we asked if Cdc53-6HA could be co-immunoprecipitated with Tem1-  
128 3Flag from cells endogenously expressing both proteins. Results showed that indeed  
129 Tem1 binds to Cdc53 in wild type cells, while interaction is severely impaired in *amn1Δ*  
130 cells (Fig. 2D, lanes 2-3), suggesting that Tem1 binding to SCF is mediated by Amn1.

131

132 Tem1 interaction with SCF<sup>Amn1</sup> suggests that Tem1 might be targeted to ubiquitin-  
133 mediated degradation and/or ubiquitin-dependent nuclear import. Therefore, we assessed  
134 if Tem1 ubiquitination (Cassani et al., 2013; Tamborrini et al., 2018) is regulated during the  
135 cell cycle and Amn1-dependent. To this end, we carried out Ni-NTA pulldowns of  
136 ubiquitinated proteins from synchronised cells overexpressing untagged or His-tagged  
137 ubiquitin, followed by western blot to detect Tem1-3HA. Since Amn1 is expressed after  
138 mitotic exit, cells were synchronised in late mitosis through the temperature-sensitive  
139 *cdc15-2* mutation and released at permissive temperature. Under these conditions, we  
140 could detect mono- and poly-ubiquitinated forms of Tem1, some of which were periodic  
141 during the cell cycle (Fig. 2E). Remarkably, cell cycle-regulated Tem1 ubiquitination  
142 peaked at 30 minutes after the late mitotic release, i.e. when Amn1-3Flag levels rose (Fig.  
143 2E), and was drastically impaired by *AMN1* deletion, suggesting that it is greatly facilitated  
144 by Amn1, presumably bound to SCF.

145 Finally, we analysed the impact of Amn1-dependent ubiquitination on Tem1 turnover. To  
146 this end, *amn1Δ* and *GAL1-AMN1* cells carrying an extra copy of *TEM1* under the control  
147 of the *GAL1* promoter were synchronised in G1 and subjected to a short pulse (30') of  
148 galactose induction, followed by glucose-mediated repression. Tem1 decay was

149 accelerated by the pulse of Amn1 (Fig. 2F), suggesting that Tem1 ubiquitination by  
150 SCF<sup>Amn1</sup> mildly stimulates Tem1 proteolysis. Consistently, *AMN1* deletion led to a two- to  
151 three-fold increase in Tem1 steady state levels (Fig. 3F), in agreement with a previous  
152 report (Wang et al., 2003).

153 **Altogether, these data suggest that** Tem1 interaction with and ubiquitination by SCF<sup>Amn1</sup>  
154 from cytokinesis to late G1 stimulates Tem1 degradation.

155

### 156 **A nuclear localization signal in Amn1, but not its F-box domain, mediates Tem1** 157 **nuclear translocation**

158 To assess if Amn1-bound SCF is involved in Tem1 nuclear import, besides its proteolysis,  
159 we deleted the F-box domain of Amn1 (aa 166-263, Fig. 3A). Indeed, F-box motifs mediate  
160 the binding of F-box proteins to SCF (Bai et al., 1996). As expected, Amn1- $\Delta$ Fbox no  
161 longer associated to Skp1 (Fig. 2B, **lane 4**). In addition, F-box deletion in Amn1 abolished  
162 the binding of Tem1 to Cdc53 without affecting Tem1-Amn1 interaction (Fig. 2C-D, **lane 4**).  
163 Thus, the F-box motif of Amn1 is necessary for Amn1 and Tem1 interaction with SCF, but  
164 not for Amn1 binding to Tem1. Interestingly, F-box deletion led to higher levels of Amn1-  
165 3Flag, as assessed by western blot (Fig. S2), consistent with the finding that Amn1  
166 proteolysis in G1 requires SCF (Wang et al., 2003). It should be noticed, however, that  
167 Amn1- $\Delta$ Fbox protein levels kept nevertheless oscillating during the cell cycle with similar  
168 kinetics to full length Amn1, suggesting that additional ubiquitin ligases control its  
169 degradation.

170 Live cell imaging of Tem1-GFP localisation in *amn1- $\Delta$ Fbox* cells showed that Tem1  
171 nuclear import was not affected (**102/102 cells**, Fig. 3B), while its levels at the daughter  
172 SPB in G1 were moderately increased (Fig. 3C). Therefore, Amn1 promotes Tem1 nuclear  
173 import independently of SCF.

174

175 We then wondered which could be the key determinants of Amn1 that mediate Tem1  
176 nuclear import. Using the cNLS-Mapper prediction software (Kosugi et al., 2009), we found  
177 a putative bipartite nuclear localisation signal (NLS) in the primary sequence of Amn1 (aa  
178 13-31, Fig. 3A). GFP-tagged Amn1 was readily detected in the nucleus of daughter cells  
179 after cytokinesis (**59/60 cells**, Fig. 3D **left**), as previously shown (Wang et al., 2003).  
180 Mutating pairs of basic residues in the NLS into alanine (aa 13-14 and 30-31), either  
181 together (Amn1-4A, Fig. 3D right) or separately (Amn1-A<sub>13</sub>A<sub>14</sub> and Amn1-A<sub>30</sub>A<sub>31</sub>, Fig. S3),  
182 prevented Amn1 nuclear translocation (**55/56 amn1-4A cells; 1/56 cells with no**



183 fluorescence), while the protein accumulated asymmetrically in the cytoplasm of the  
184 daughter cell. Thus, the identified NLS is crucial for Amn1 nuclear localisation. The lack of  
185 Amn1 diffusion between mother and bud compartment is consistent with the notion that  
186 Amn1 synthesis occurs after cytokinesis, i.e. after complete separation of mother and bud  
187 cytoplasm. Importantly, the *amn1-4A* mutant allele did neither affect Amn1 binding to Skp1  
188 (Fig. 2B, lane 5) nor Tem1 interaction with Amn1 and Cdc53 (Fig. 2C, lane 3; Fig. 2D, lane  
189 5).

190 In agreement with an escorting function of Amn1 in Tem1 nuclear import, Tem1 was not  
191 translocated into the nucleus of *amn1-4A* cells at any cell cycle stage (70/70 cells, Fig. 3E  
192 and S4). Moreover, Tem1 completely disappeared from the daughter cell SPB during G1  
193 (i.e. around the time of cytoplasmic accumulation of Amn1, Fig. 3E and S4), indicating that  
194 binding to Amn1 evicts Tem1 from SPBs even in the absence of nuclear translocation.  
195 Remarkably, *amn1-4A* cells displayed even lower levels of Tem1 at the daughter SPB in  
196 G1 than wild type cells (Fig. 3C). This is likely a consequence of Amn1 failure to enter the  
197 nucleus, which increases its window of opportunity for dislodging Tem1 from the SPB.

198

199 We took advantage of the *amn1-4A* mutant to assess the possible contribution of Tem1  
200 nuclear import on its proteolysis. By analysing Tem1 protein levels by western blot, we  
201 found that F-box deletion or NLS mutation individually caused a mild stabilization of Tem1  
202 in G1, while deletion of both motifs together led to an additive increase in Tem1 levels,  
203 albeit not as pronounced as in *amn1Δ* cells (Fig. 3F). Conversely, *AMN1* overexpression  
204 from the galactose-inducible *GAL1* promoter (*GAL1-AMN1*) dramatically decreased Tem1  
205 protein levels in an F-box-dependent manner (Fig. 3G). Thus, nuclear import and binding  
206 to SCF both contribute to Tem1 proteolysis.

207

208 In summary, Amn1 inhibits Tem1 at SPBs by two different mechanisms: SCF-mediated  
209 protein degradation and SCF-independent nuclear translocation (Fig. 4). Whether Tem1  
210 has functions independent of MEN inside the nucleus is an intriguing possibility that  
211 deserves further investigation.

212 Given that Tem1 gets activated at SPBs, the dual mode of action of Amn1 may ensure  
213 rapid Tem1 inactivation after cytokinesis. Competition between Amn1 and Cdc15 for their  
214 association with Tem1 (Wang et al., 2003) could provide an additional mode of Tem1  
215 downregulation.

216 Since *AMN1* expression requires the transcription factor Ace2, which in turn enters the  
217 nucleus upon MEN-dependent activation of Cdc14, Tem1 eviction from the daughter SPB  
218 and degradation are only possible after Tem1 has fulfilled its MEN-promoting functions. On  
219 the other hand, being Amn1 itself and Ace2 targets of SCF<sup>Amn1</sup>, their clearance in G1 re-  
220 sets the stage for Tem1 re-accumulation in the following S and M phases. Thus, this  
221 sophisticated cell cycle circuit **contributes to** the ordered relay of MEN-regulated  
222 processes.  
223

## 224 MATERIALS AND METHODS

225

### 226 Yeast strains, plasmids and growth conditions

227 All yeast strains (Table S1) are congenic to or at least four times backcrossed to W303

228 (*ade2-1, trp1-1, leu2-3,112, his3-11,15, ura3*).

229 One-step tagging techniques were used to generate 3HA-, yeGFP-, sfGFP, mScarlet-I and

230 mCherry-tagged proteins at the C-terminus. The **Tem1-yeGFP construct had been**

231 **previously described (Scarfone et al., 2015)**. A Yiplac211 plasmid bearing *mCherry-PUS1*

232 (gift from Maria Moriel Carretero) was integrated at the *ura3* locus after cutting with *Apal*.

233 The *shs1-GFP*-bearing BYP6904 plasmid was obtained from the National BioResource

234 Project (Yeast) (<https://yeast.nig.ac.jp/yeast>) and used to generate the *shs1::LEU2::SHS1-*

235 *GFP* strain after cutting with *BglIII* and integration at the *SHS1* locus. *GAL1-TEM1* and

236 *GAL1-AMN1* strains were generated by inserting a *natNT2-GAL1* cassette upstream the

237 ATG at the *TEM1-3HA* and *AMN1-3Flag* locus, respectively. Since *TEM1* is an essential

238 gene, the *natNT2-GAL1* cassette was integrated into a strain that carried an extra copy of

239 *TEM1* on a centromeric plasmid. Strains deleted of the entire *AMN1* coding sequence

240 were obtained by insertion of the *KanMX*, *K.I.URA3* or *C.a.URA3* cassettes by one-step

241 transformation. A pRS313-*AMN1* plasmid (pSP1402) was constructed by inserting a DNA

242 fragment covering the entire coding sequence of *AMN1* with 500 bp of 5' UTR and 178 bp

243 of 3' UTR, amplified by PCR from genomic DNA of a wild type strain. Lys13 and Arg14 of

244 *Amn1* were both mutated to Ala by inserting a synthetic DNA duplex made of oligos

245 MP902 and MP903 between the *MluI* and *EcoRI* sites in the *AMN1* gene. Lys30 and

246 Lys31 of *Amn1* were both mutated to Ala by inserting a synthetic DNA duplex carrying

247 these mutations (Genecust) between the *EcoRV* and *EcoRI* sites in the *AMN1* gene. The

248 deletion of the F-box domain of *Amn1* (amino acids 163 to 263) was obtained by inverse

249 PCR using oligos MP1037 and MP1038. Mutated *AMN1* alleles were transplaced at the

250 *AMN1* locus by replacing the *URA3* marker in *amn1::K.I.URA3* or *amn1::C.a.URA3* strains

251 followed by counter-selection on FoA-containing plates. The pRS413-*TEM1* plasmid

252 (pSP526) contains the entire coding sequence for *TEM1* and 524 bp of 5' UTR.

253 Yeast cultures were grown at 25-30°C, in either SD medium (6.7 g/L yeast nitrogen base

254 without aminoacids), supplemented with the appropriate nutrients, or YEP (1% yeast

255 extract, 2% bactopectone, 50 mg/L adenine) medium. Raffinose was supplemented to 2%

256 (SD-raffinose or YEPR), glucose to 2% (SD-glucose or YEPRD), and galactose to 1% (SD-

257 raffinose/galactose or YEPRG). Cells were synchronized in G1 by alpha factor (4 µg/mL)

258 in YEP medium containing the appropriate sugar at 25°C. G1 arrest was monitored under  
259 a transmitted light microscope and cells were released in fresh medium (typically after  
260 120–135 min of alpha factor treatment) at 30°C, unless otherwise specified, after being  
261 collected by centrifugation at 2000g followed by one wash with YEP containing the  
262 appropriate sugar.

263

#### 264 **Detection of ubiquitin conjugates**

265 Analysis of 6His-tagged ubiquitin Tem1 conjugates was performed as previously described  
266 (Tamborrini et al., 2018).

267

#### 268 **FACS analysis of DNA contents**

269 Yeast cells were collected and treated as described (Benzi et al., 2020) and analysed on a  
270 ACEA NovoCyte cytometer.

271

#### 272 **Protein extracts, immunoprecipitations and western blotting**

273 For TCA protein extracts, 10–15 mL of cell culture in logarithmic phase (OD<sub>600</sub> = 0.5-1)  
274 were collected by centrifugation at 2000g, washed with 1 mL of 20% TCA and  
275 resuspended in 100 µL of 20% TCA before breakage of cells with glass beads (diameter  
276 0.5–0.75 mm) on a Vibrax VXR (IKA). After addition of 400 µL of 5% TCA, lysates were  
277 centrifuged for 10 min at 845g. Protein precipitates were resuspended in 100 µL of 3X  
278 SDS sample buffer (240 mM Tris-Cl pH6.8, 6% SDS, 30% glycerol, 2.28 M β-  
279 mercaptoethanol, 0.06% bromophenol blue), denatured at 99°C for 3 min and loaded on  
280 SDS-PAGE after elimination of cellular debris by centrifugation (5 min at 20000  
281 g).

282 Native yeast protein extracts for immunoprecipitations were performed from cell pellets  
283 (equivalent to ~50 OD<sub>600</sub>), after centrifugation at 2000g and washing with 1 mL of cold 10  
284 mM Tris-Cl pH 7.5. Cells were then lysed using glass beads in a Bullet Blender in 25 mM  
285 Tris-Cl pH 7.4, 100 mM NaCl, 2 mM EDTA, 1 mM DTT, 0.1% IGEPAL containing a cocktail  
286 of protease inhibitors (Complete EDTA-free Roche) and phosphatase inhibitors  
287 (PhosSTOP Roche). Lysates were cleared at 20000g for 10 min at 4 °C and incubated on  
288 a nutator for 2h at 4 °C with 15 µL of protein-G Dynabeads and anti-Flag-M2 antibodies.  
289 After washing the beads three times, proteins were eluted in 50 mM Tris-HCl pH 8.3, 1 mM  
290 EDTA, 0.1% SDS containing 0.5 mg/ml of Flag peptide.

291 For western blotting, proteins were wet-transferred on Protran membranes (Schleicher and  
292 Schuell). Total proteins were revealed by amido-black staining and quantified with ImageJ  
293 after scanning the membranes. Specific proteins were detected with monoclonal anti-HA  
294 12CA5 (AgroBio, 1:5000) or monoclonal anti-Flag M2 (Sigma F3165, 1:5000). Antibodies  
295 were diluted in 5% low-fat milk (Regilait) dissolved in TBST. Secondary antibodies were  
296 purchased from GE-Healthcare and proteins were detected by a luminol/p-coumaric acid  
297 enhanced chemiluminescence system. Membranes were imaged with a Amersham  
298 Imager-600 and protein bands were quantified with ImageJ.

299

### 300 **Fluorescence microscopy**

301 Imaging of Tem1 at SPBs in cells arrested in G1 with alpha factor was performed after  
302 fixation in 4% PFA. Still digital images were taken with an oil immersion 100X 1.4 HCX  
303 PlanApochromat objective (Zeiss) with a Coolsnap-HQ2 CDD camera (Photometrics)  
304 mounted on a Zeiss AxioimagerZ1 fluorescence microscope and controlled by the  
305 MetaMorph imaging system software. Z stacks containing 18 planes were acquired with a  
306 step size of 0.3  $\mu\text{m}$  and max-projected using ImageJ. Fluorescence intensities were  
307 measured with ImageJ. SPB particles were identified on the Spc42-mCherry images with  
308 the ImageJ Analyze Particles tool after applying a threshold. Maximum pixel values  
309 present in these SPB particles were extracted in both Spc42-mCherry and Tem1-yeGFP  
310 images. Background values for both channels were determined as the mean pixel value of  
311 several regions outside SPB particles. The  $\log_2$  of Tem1 signals was finally plotted  
312 according to the following equation:  $\log_2((\text{SPB}^{\text{MAX}} - \text{Bkgnd})^{\text{GFP}} / (\text{SPB}^{\text{MAX}} - \text{Bkgnd})^{\text{mCherry}})$   
313 (Fig. S1B, 3C). For time-lapse video microscopy, cells were mounted on 0.8% agarose  
314 pads in SD medium on Fluorodishes and filmed at controlled temperature (30°C) with a  
315 100X 1.49 NA oil immersion objective mounted on a Nikon Eclipse Ti microscope  
316 equipped with an EMCCD Evolve-512 Camera (Photometrics) and iLAS2 module (Roper  
317 Scientific) and controlled by Metamorph. Z stacks of 13 planes were acquired every 3-4  
318 min with a step size of 0.4  $\mu\text{m}$  in HILO mode. Z stacks were max-projected with ImageJ.  
319 Alternatively, time-lapse video microscopy was performed using a 100X Plan Apo lambda  
320 1.45 NA objective mounted on a Dragonfly Andor spinning disk equipped with dual camera  
321 for 2-channel simultaneous acquisition and coupled to a Ti2 Nikon microscope (Fig 3C).  
322 Tem1 signals at SPBs were quantified as above, except that the background was  
323 calculated for each individual SPB as the mean pixel value in the vicinity of the SPB.

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330

331 **COMPETING INTERESTS**

332 No competing interests declared.

333

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463

464 **FIGURE LEGENDS**

465

466 **Figure 1. Amn1 escorts Tem1 into the nucleus at the M/G1 transition.**

467 **A.** Cells expressing Tem1-yeGFP and mCherry-Pus1 were imaged every 4 minutes.  
468 Arrows indicate nuclear Tem1. **Tem1-yeGFP nuclear signals (mean  $\pm$  s.d.) were plotted**  
469 **(graph) relative to the onset of anaphase (t=0; n=24).** **B.**  $\Delta amn1$  cells expressing Tem1-  
470 yeGFP were imaged as in A. **C.** Wild type (wt) and  $\Delta amn1$  cells co-expressing Tem1-  
471 yeGFP, Shs1-GFP and Spc42-mCherry (SPB marker) were imaged every 3 minutes.  
472 Arrows indicate nuclear Tem1. Mean values (curves) and s.d. (shades) of Tem1-yeGFP  
473 signals normalised to Spc42-mcherry were plotted (graphs) relative to septin disassembly  
474 (t=0; n= 28-54). A.U.: Arbitrary units. **D.** Cells co-expressing Amn1-sfGFP and Tem1-  
475 mScarlet-I were imaged as in A. Arrows indicate Amn1 and Tem1 nuclear entry. DIC:  
476 Differential Interference Contrast. Scale bars: 5  $\mu$ m. **m: mother cell; d: daughter cell.**  
477

478 **Figure 2. SCF<sup>Amn1</sup> promotes Tem1 ubiquitination and degradation.**

479 **A-D.** Amn1-3Flag (A,B,C), its mutant variants ( $\Delta$ Fbox, 4A) (B,C) or Tem1-3Flag (D) were  
480 immunoprecipitated from asynchronously growing cells. Immunoprecipitates (IP-Flag)  
481 were probed with an anti-HA antibody to detect Cdc53-6HA (A,D), Skp1-6HA (B) or Tem1-  
482 3HA (C). **Note that Tem1-3HA reproducibly runs as a doublet (see also Fig. 2E-F, 3F-G).**  
483 **E.** *cdc15-2* temperature-sensitive cells overexpressing untagged or 6His-tagged ubiquitin  
484 were grown at 25°C (async) and shifted to 37°C for 3 hours (late mitotic arrest). After  
485 release in fresh medium at 25°C, cells were collected at the indicated times for Ni-NTA  
486 pulldowns of ubiquitinated proteins (His-PD) and FACS analysis of DNA contents  
487 (righthand histograms). Presence of ubiquitinated Tem1-3HA was assessed with an anti-  
488 HA antibody. Wild type cells co-expressed Amn1-3Flag to correlate Tem1 ubiquitination  
489 with Amn1 levels throughout the experiment. **The experiment was repeated three times**  
490 **with similar results.** Note that the bulk of DNA replication occurs at 45 minutes after  
491 release (righthand histograms; **1C: pre-replicative DNA content; 2C: post-replicative DNA**  
492 **content**). The appearance of a 4C post-replicative peak of DNA content is due to the cell  
493 separation defect of *cdc15-2* cells (Piatti et al., 1996). **F.**  $\Delta amn1$  and *GAL1-AMN1* cells  
494 bearing an extra copy of **3HA-tagged TEM1** under the control of the *GAL1* promoter, were  
495 grown in YEPR and synchronised in G1 with alpha factor. After 30 minutes induction with  
496 1% galactose, cells were transferred to glucose-containing medium to switch off the *GAL1*  
497 promoter (*GAL1-AMN1* and *GAL1-TEM1-3HA*). **Protein levels of Tem1-3HA (upper and**

498 lower band) were quantified by western blot and normalised to the total protein levels in  
499 the samples (amido-Black staining) for plotting (graph). The experiment was repeated  
500 three times with similar results. A.U.: Arbitrary units. WCE: whole cell extracts.

501

502 **Figure 3. The NLS of Amn1, but not its F-box domain, is required for Tem1 nuclear**  
503 **import.**

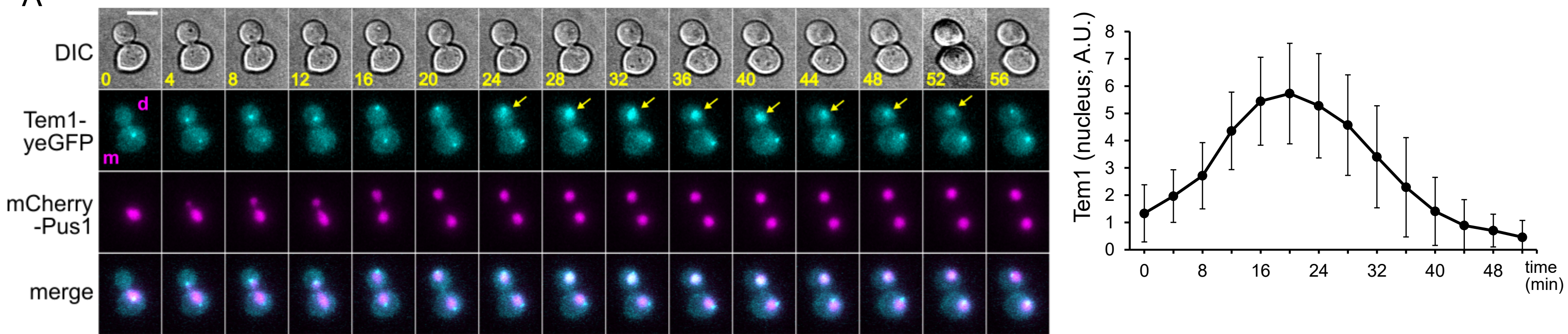
504 **A.** Aminoacid sequence of Amn1 Fbox domain (Willems et al., 2004) and NLS (Kosugi et  
505 al., 2009). Conserved residues are highlighted in blue and red, respectively. **B.** Tem1-  
506 yeGFP was imaged every 4' in *amn1-ΔFbox* cells. Yellow arrows: SPB-localised Tem1-  
507 yeGFP; magenta arrows: nuclear Tem1-yeGFP. **C.** Live cells co-expressing Tem1-yeGFP,  
508 Shs1-GFP (septin) and Spc42-mCherry were imaged every 3 minutes. Tem1-yeGFP  
509 signals were quantified at the bud SPB 12' after mitotic exit, as assessed by disassembly  
510 of Shs1-GFP at the bud neck, and normalised relative to Spc42-mCherry. Medians, first  
511 and third quartiles are shown in red. *p*-values were obtained using a Mann-Whitney test.  
512 **D.** Amn1-yeGFP and Amn1-4A-yeGFP were imaged every 4'. Arrows indicate nuclear  
513 Amn1. **E.** Tem1-yeGFP and Spc42-mCherry were imaged every 4' in *amn1-4A* cells. Note  
514 the decrease of Tem1-yeGFP fluorescence at the bud SPB (times 16'-48'), in the absence  
515 of nuclear import. **F-G.** Protein levels of Tem1-3HA (upper and lower band) were  
516 quantified by western blot in total extracts from G1-arrested cells (F) or from  
517 asynchronously growing cells after overnight galactose induction (G). Protein levels were  
518 plotted (graph) relative to total protein levels (Coomassie staining). A.U. Arbitrary Units.  
519 N=3-5. Error bars: SD. DIC: Differential Interference Contrast. Scale bars: 5 μm. m: mother  
520 cell; d: daughter cell.

521

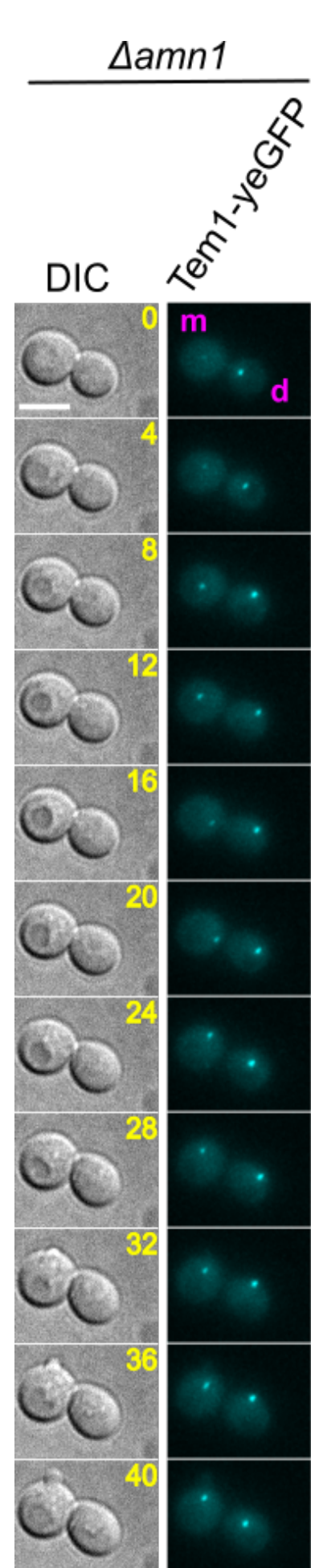
522 **Figure 4. Dual mode of Tem1 regulation by Amn1.**

523 Tem1 is active at SPBs, where it recruits and activates MEN (not depicted). Amn1 evicts  
524 Tem1 from the bud SPB through direct binding (1) and escorts it into the nucleus (2).  
525 Tem1 also associates with SCF<sup>Amn1</sup>, presumably in the nucleus and in the cytoplasm,  
526 which leads to its degradation (3).

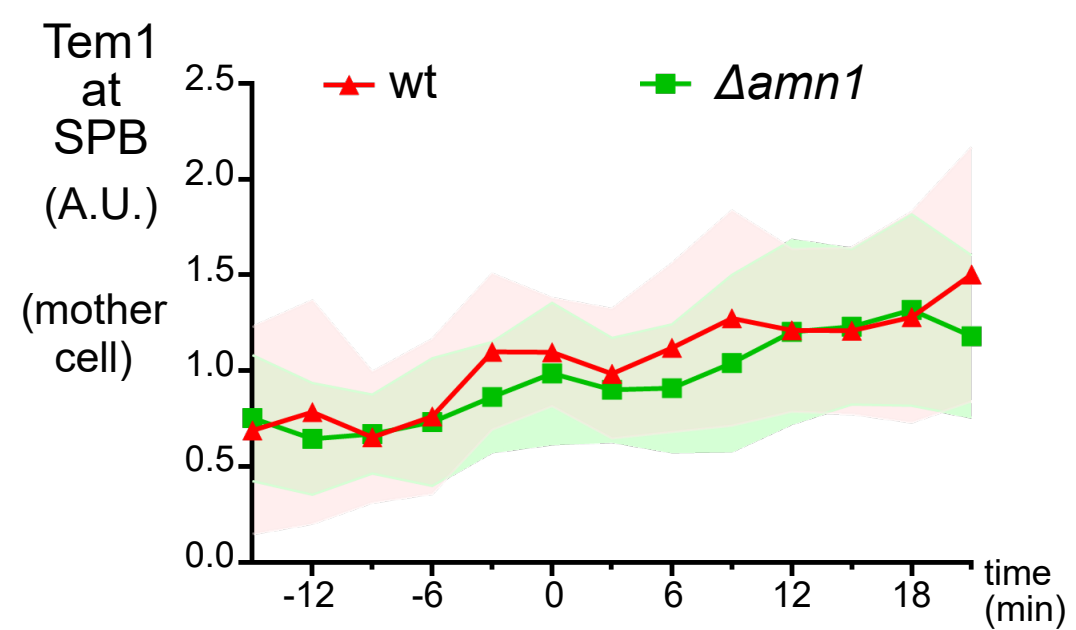
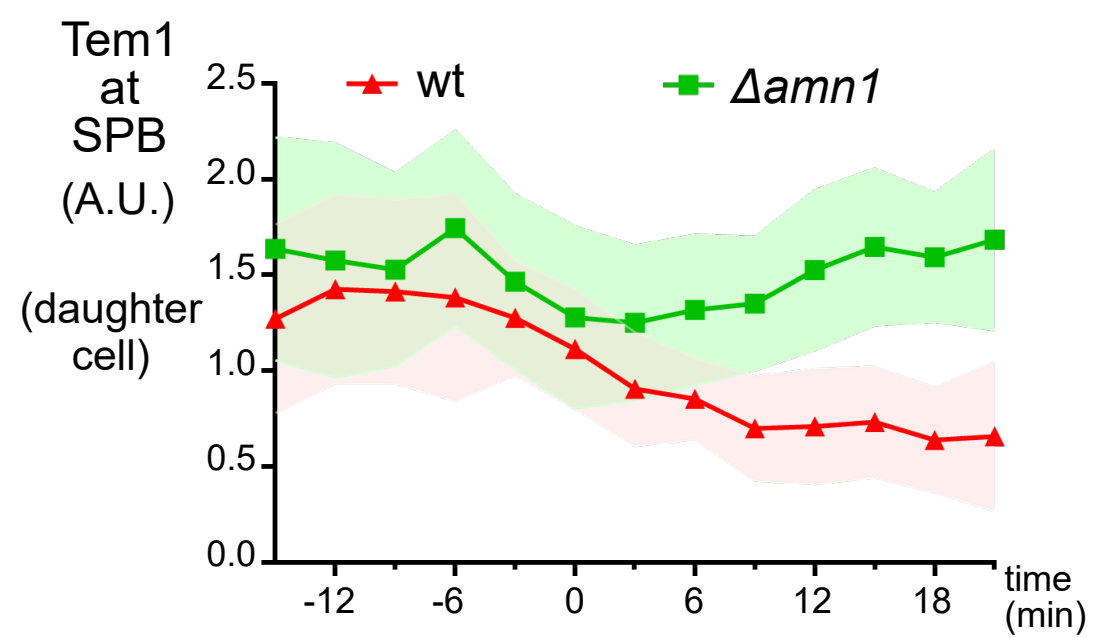
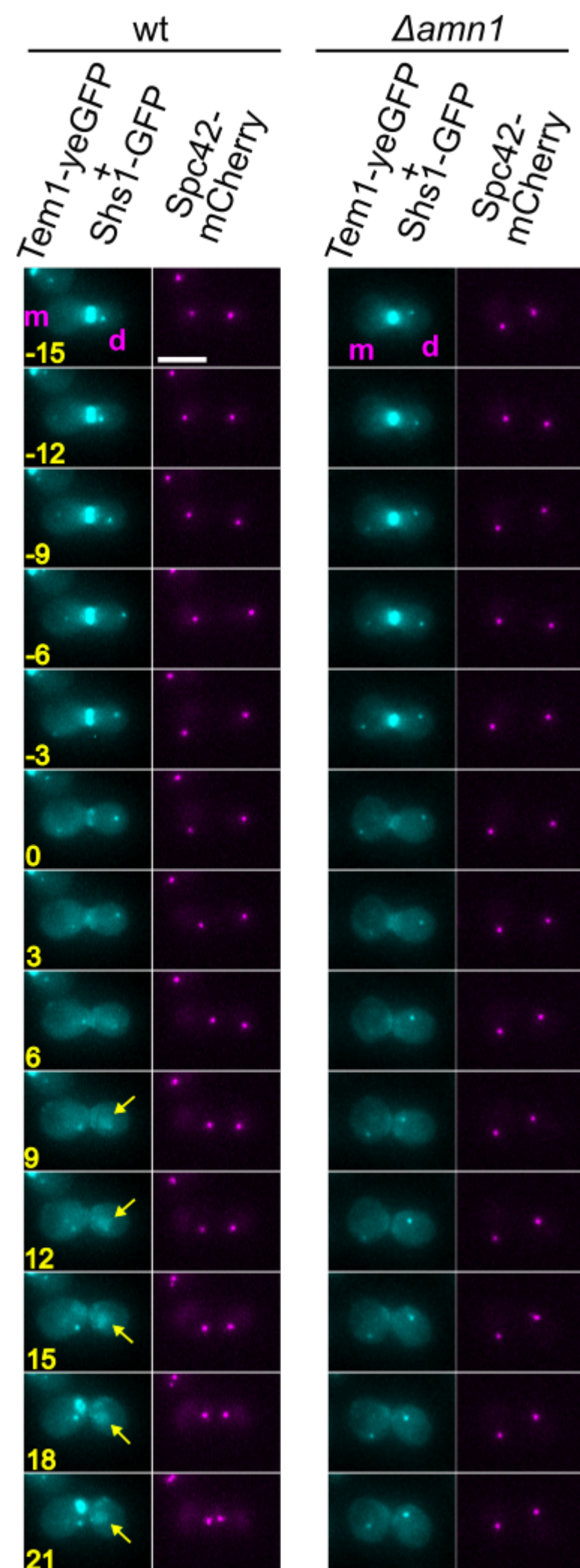
A



B



C



D

